

## Brief Review

# Adrenergic Receptors

## Models for Regulation of Signal Transduction Processes

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Adrenergic receptors are prototypic models for the study of the relations between structure and function of G protein-coupled receptors. Each receptor is encoded by a distinct gene. These receptors are integral membrane proteins with several striking structural features. They consist of a single subunit containing seven stretches of 20–28 hydrophobic amino acids that represent potential membrane-spanning  $\alpha$ -helices. Many of these receptors share considerable amino acid sequence homology, particularly in the transmembrane domains. All of these macromolecules share other similarities that include one or more potential sites of extracellular N-linked glycosylation near the amino terminus and several potential sites of regulatory phosphorylation that are located intracellularly. By using a variety of techniques, it has been demonstrated that various regions of the receptor molecules are critical for different receptor functions. The seven transmembrane regions of the receptors appear to form a ligand-binding pocket. Cysteine residues in the extracellular domains may stabilize the ligand-binding pocket by participating in disulfide bonds. The cytoplasmic domains contain regions capable of interacting with G proteins and various kinases and are therefore important in such processes as signal transduction, receptor-G protein coupling, receptor sequestration, and down-regulation. Finally, regions of these macromolecules may undergo posttranslational modifications important in the regulation of receptor function. Our understanding of these complex relations is constantly evolving and much work remains to be done. Greater understanding of the basic mechanisms involved in G protein-coupled, receptor-mediated signal transduction may provide leads into the nature of certain pathophysiological states. (*Hypertension* 1990;15:119–131)

The endogenous catecholamines epinephrine and norepinephrine are thought to modulate blood pressure homeostasis through at least four potential mechanisms. First, catecholamines affect blood pressure through actions on the central nervous system.<sup>1</sup> Second, they influence blood pressure by directly controlling cardiovascular tone.<sup>2–4</sup> Third, they modulate renal sodium flux, primarily in the proximal tubule.<sup>5–8</sup> Fourth, catecholamines can affect renin release and the renin-angiotensin-aldosterone system.<sup>9</sup> The catecholamines, as well as various synthetic congeners, initiate these actions by specifically interacting with membrane-bound receptor macromolecules. The interaction of catecholamines with their receptors typically fulfills several

criteria. Binding to the receptors is rapid, saturable, stereoselective, reversible, and of high affinity. Furthermore, occupancy of the receptor by the appropriate agonist leads to generation of intracellular second messengers and culminates in a physiological response. As with many other receptors, those for catecholamines are linked to specific effector systems through intermediary signal-transducing proteins. These are termed guanine nucleotide regulatory proteins, or “G proteins” because they bind and hydrolyze the guanine nucleotide guanosine triphosphate (GTP).<sup>10</sup>

Catecholamine (adrenergic) receptors can be subdivided into several distinct categories, based on pharmacological specificity and physiological actions. These include the  $\alpha_1$ -,  $\alpha_2$ -,  $\beta_1$ -, and  $\beta_2$ -adrenergic receptor subtypes. These subtypes may be subdivided yet further into sub-subtypes such as  $\alpha_{2A}$  and  $\alpha_{2B}$ ,<sup>11</sup> and  $\alpha_{1A}$  and  $\alpha_{1B}$ ,<sup>12–14</sup> and perhaps others.<sup>15–17</sup> These distinct categories of receptors differ not only in their specificity for various ligands but also in their specificity for coupling to G proteins and thereby different second messenger systems. For instance, agonist activation of either the  $\beta_1$ - or  $\beta_2$ -adrenergic receptor subtype classically leads to the generation of cyclic adenosine monophosphate (cAMP) by stimulating

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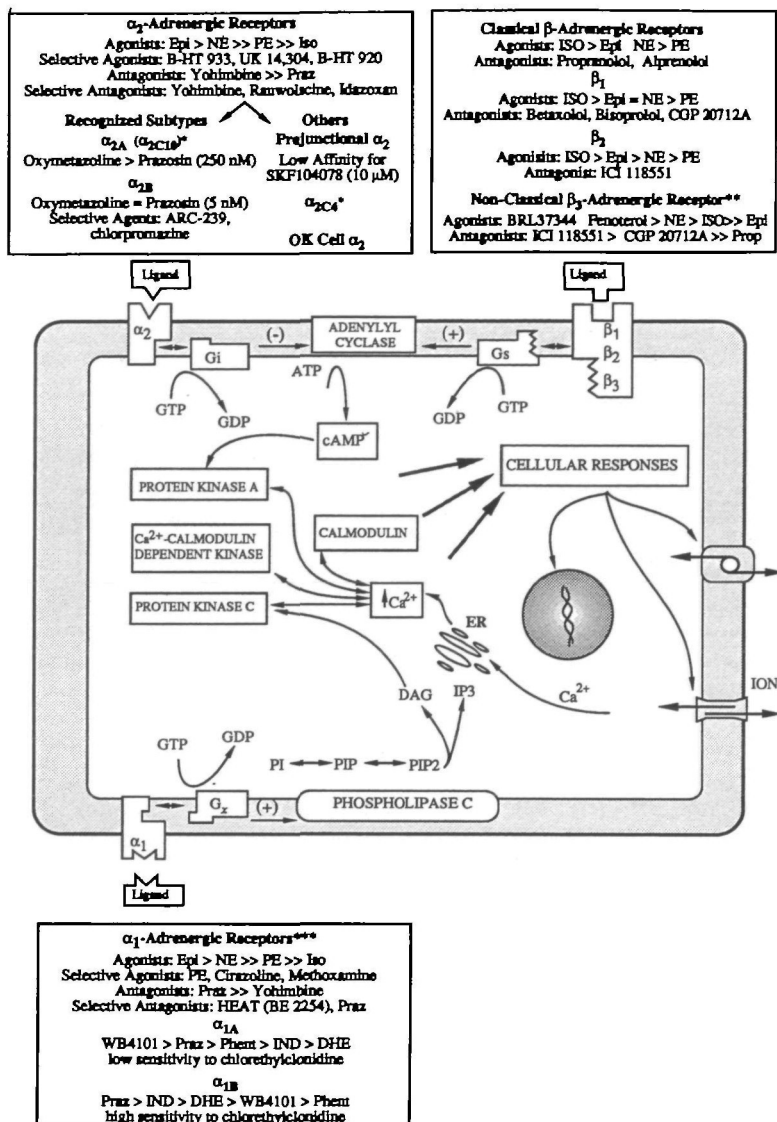


FIGURE 1. Schematic representation of cell signal transduction pathways linked to adrenergic receptor subtypes. Epi, epinephrine; NE, norepinephrine; PE, phenylephrine; Iso, isoproterenol; Praz, prazosin; IND, indanidine; DHE, dihydroergocryptine; Phent, phentolamine; DAG, diacylglycerol; IP<sub>3</sub>, inositol trisphosphate; ER, endoplasmic reticulum; cAMP, cyclic adenosine 3',5'-monophosphate; GTP, guanosine triphosphate; GDP, guanosine diphosphate; Prop, propranolol; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-monophosphate. \*Indicates that these two receptor subtypes have also been demonstrated to couple to PIP<sub>2</sub> hydrolysis when expressed in hamster pulmonary fibroblast cells.<sup>18</sup> \*\*Indicates that these data are based on single study of recombinant receptors expressed in CHO cells.<sup>17</sup> \*\*\*Indicates that α<sub>1B</sub>-adrenergic receptors are thought to couple primarily to PIP<sub>2</sub> hydrolysis, whereas α<sub>1A</sub>-adrenergic receptors are thought to couple in an unknown fashion to dihydropyridine-sensitive Ca<sup>2+</sup> channels (see References 12 and 13 for reviews).

the enzyme adenylyl cyclase; this pathway is mediated by the G protein G<sub>s</sub>. Conversely, agonist activation of α<sub>2</sub>-adrenergic receptor leads primarily to inhibition of adenylyl cyclase via a distinct G protein, G<sub>i</sub>.<sup>11</sup> In contrast, α<sub>1</sub>-adrenergic receptor activation leads to generation of the second messengers diacylglycerol and inositol trisphosphate by stimulating the enzyme phospholipase C<sup>18</sup>; this pathway is mediated by a pertussis toxin-insensitive G protein termed G<sub>q</sub>, although this term is not universally accepted (G<sub>x</sub> or G<sub>z</sub> may be more appropriate).

Agonist occupancy of G protein-coupled receptors or generation of these various second messengers can activate a cascade of events leading to, for example, the activation of specific kinases and subsequent phosphorylation of proteins<sup>19</sup> (e.g., the cAMP-dependent protein kinase protein kinase A [PKA], the Ca<sup>2+</sup> phospholipid-dependent protein kinase protein kinase C [PKC]),<sup>20,21</sup> release of intracellular Ca<sup>2+</sup> stores,<sup>22</sup> activation of ion channels<sup>23,24</sup> or pumps,<sup>8</sup> release of arachidonic acid,<sup>25</sup> and gene

transcription (see Figure 1). The diversity of receptor subtypes and effector systems linked to the adrenergic receptors allows for a wide array of physiological responses and a complex web of potential feedback mechanisms. Moreover, recent data suggest that individual receptor subtypes may be capable of coupling to different G proteins<sup>18,26,27</sup> further illustrating the complexity of receptor-activated signaling networks (discussed below).

Although receptors for many hormones, peptides, neurotransmitters, light,<sup>28</sup> and perhaps even odorant molecules<sup>29</sup> are coupled to G proteins, the adrenergic receptors are perhaps the most thoroughly characterized. As such, the adrenergic receptor family may serve as a model for the study of the superfamily encompassing all G protein-coupled receptors. Three of the four main subtypes (α<sub>1</sub>, α<sub>2</sub>, and β<sub>2</sub>) have been purified to homogeneity and reconstituted into phospholipid vesicles with G proteins.<sup>30</sup> The genes or complementary DNAs (cDNAs) of at least six subtypes have been isolated, sequenced, and expressed

TABLE 1. Characteristics of the Second Messenger Linkages of the Cloned Adrenergic Receptors

Receptor	cAMP			PI		
	Response	EC <sub>50</sub>	G Protein	Response	EC <sub>50</sub>	G Protein
$\beta_2$	↑ ↑	100 nM	G <sub>s</sub>	...	...	...
$\beta_3$	↑ ↑	3.9 nM	G <sub>s</sub>	...	...	...
$\alpha_1$	↑	300 nM	Indirect?	↑ ↑	300 nM	PTx-Insensitive "G <sub>x</sub> "
$\alpha_{2A}$	↓ ↓	100 nM	PTx-Sensitive G <sub>i</sub>	↑	10 $\mu$ M	PTx-Sensitive ?G <sub>i</sub> , "G <sub>x</sub> "?
$\alpha_{2B}$	↓ ↓	10 nM	PTx-Sensitive G <sub>i</sub>	↑	1 $\mu$ M	PTx-Sensitive ?G <sub>i</sub> , "G <sub>x</sub> "?

Experiments were performed in various mammalian cell lines or *Xenopus* oocytes transiently or stably expressing recombinant adrenergic receptors.<sup>17,18,46-49,55-57</sup> cAMP, cyclic adenosine 3',5'-monophosphate; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; EC<sub>50</sub>, dose at which half maximal effect occurs; PTx, pertussis toxin. Agonists used for the  $\beta$ -adrenergic receptors and  $\alpha$ -adrenergic receptors were isoproterenol and epinephrine, respectively.

in mammalian cell lines.<sup>16,17,31-38</sup> Finally, their functional regulation has been extensively studied by covalent modification,<sup>19,39-41</sup> mutagenesis,<sup>42-47</sup> and chimeric receptor construction.<sup>48,49</sup> In this review, we will discuss the relations of adrenergic receptor structure to such diverse functions as ligand binding, G protein activation, desensitization, posttranslational modifications, and other regulatory phenomena.

#### Classification of Adrenergic Receptors

Adrenergic receptors were initially subdivided by Ahlquist<sup>50</sup> into two major subtypes,  $\alpha$  and  $\beta$ , based on their pharmacological characteristics. This model was subsequently refined to include subtypes,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_1$ , and  $\beta_2$ , based on functional correlates and pharmacological specificity.<sup>51-53</sup> More recent proposals subdivide the  $\alpha_2$ -adrenergic receptor into at least two subtypes (A,B) based on pharmacological differences in affinities for prazosin, oxymetazoline, idazoxan, and SKF104078.<sup>11,15</sup> Clearly, the cloned human  $\alpha_{2C10}$ -receptor (named for the location of its gene on chromosome 10) corresponds to the pharmacologically defined platelet  $\alpha_{2A}$ -adrenergic receptor.<sup>11,36</sup> However, other  $\alpha_2$ -adrenergic receptors such as that expressed in OK cells, the cloned human  $\alpha_{2C4}$ -adrenergic receptors (named for the location of its gene on chromosome 4), as well as "prejunctional"  $\alpha_2$ -adrenergic receptors, which have a low affinity for the compound SKF104078, do not fit into this classification scheme.<sup>11,15,16</sup> A similar heterogeneity of  $\alpha_1$ -adrenergic receptors (A and B subtypes) has been proposed based on criteria such as relative potencies of a series of antagonists, differential sensitivity to the agonist methoxamine or to the alkylating agent chlor-ethylclonidine, and differential requirements for calcium in the generation of second messengers<sup>12,13</sup> (see Figure 1).

Biochemically, adrenergic receptors couple to several well-characterized signal transduction systems. Both  $\beta_1$ - and  $\beta_2$ -adrenergic receptors stimulate adenylyl cyclase, as does the recently cloned  $\beta_3$ -adrenergic receptor,<sup>17</sup> whereas  $\alpha_2$ -adrenergic receptors have been classically shown to inhibit adenylyl cyclase in tissues where they are expressed.<sup>4</sup> However, it is evident that

many of these so-called cyclase-inhibitory receptors can also couple to a positive modulation of ion channels<sup>54</sup> or phospholipase C.<sup>26,27</sup> The  $\alpha_1$ -adrenergic receptor on the other hand is coupled to phospholipase C; however, there is evidence to suggest that  $\alpha_1$ -adrenergic receptor can also couple to arachidonic acid metabolism, presumably via phospholipase A<sup>25</sup> and to calcium channels via an unknown mechanism.<sup>12-14</sup> It is not known whether these actions are mediated by multiple receptor subtypes, or via a single subtype linked to different effector systems, although it seems apparent that at least two subtypes of  $\alpha_1$ -adrenergic receptors exist. The characteristics of the second messenger systems of the cloned adrenergic receptors are presented in Table 1. As more receptor genes are cloned, expressed, and reconstituted, the complex interactions between the various components of signaling pathways linked to G protein-coupled receptors will be elucidated.

It is now clear that many of the pharmacological subtypes of adrenergic as well as other receptors are encoded by separate genes (see Figure 2) and that the pharmacological and biochemical diversity observed in various tissues and organisms is typically not due to posttranslational modification of the products of a single gene. With this emerging heterogeneity, it is likely that the future classification of adrenergic and other G protein-coupled receptors should be based on a combination of pharmacology, specificity of coupling to G proteins or effector systems, physiological effects, as well as the chromosomal location of the receptor genes.

#### Structure of G Protein-Coupled Receptors

As depicted in Figure 3, G protein-coupled receptors are integral membrane proteins with several striking structural features. These receptors consist of a single subunit containing seven stretches of 20-28 hydrophobic amino acids, which represent potential membrane-spanning  $\alpha$ -helices. The adrenergic receptors share considerable amino acid sequence identity, with the highest homology residing within the membrane-spanning domains and, to a

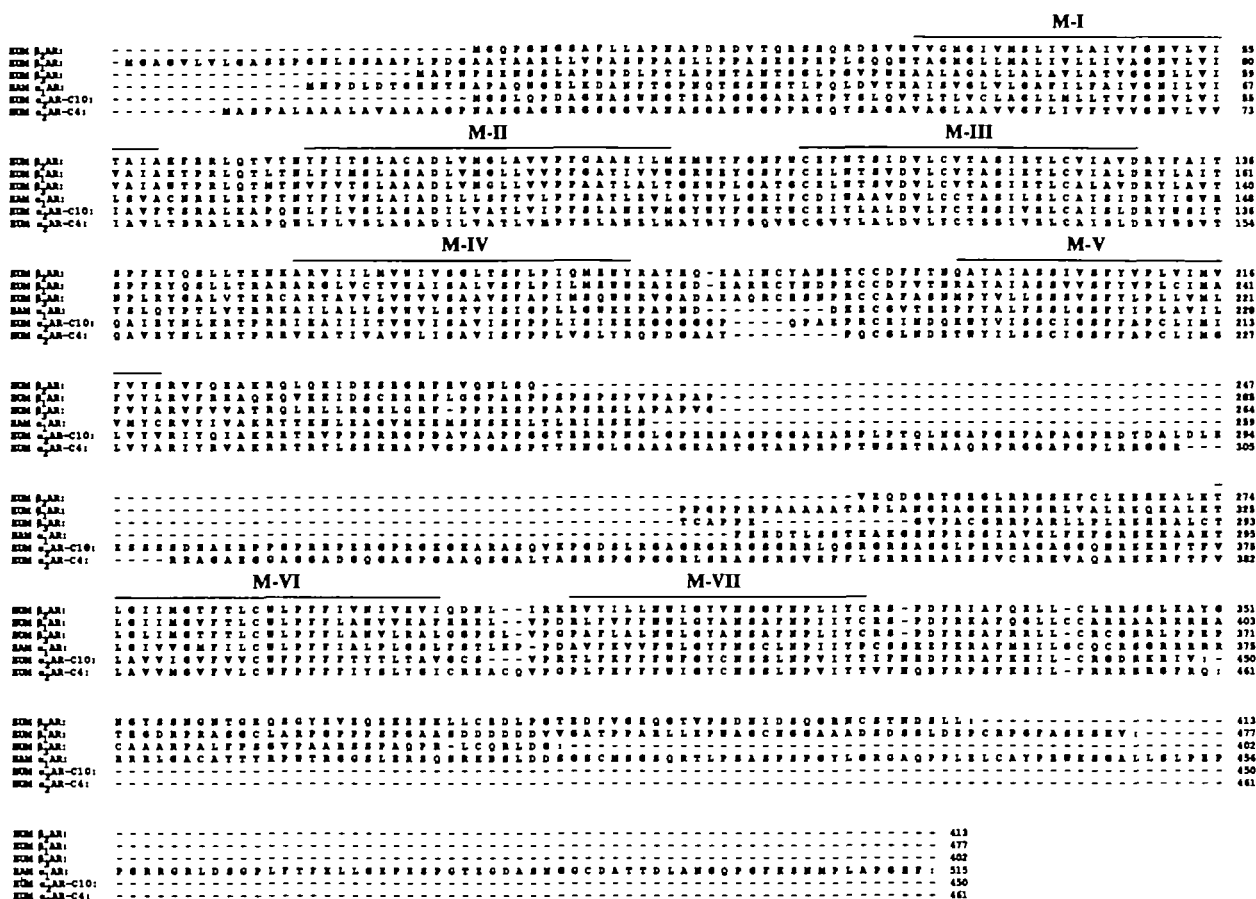


FIGURE 2. Amino acid sequence comparison of cloned adrenergic receptors. Residue 1 of each receptor is an extracellular amino terminal methionine. Putative membrane spanning domains (seven for each receptor) are represented by bars. These membrane-spanning domains were determined by hydrophobicity analyses as described by Kyte and Doolittle.<sup>58</sup>  $\alpha_{2C10}$ -Adrenergic receptor (human platelet) encodes the  $\alpha_{2A}$ -adrenergic receptor;  $\alpha_{2C4}$ -adrenergic receptor gene encodes an  $\alpha_2$ -adrenergic receptor subtype that does not currently fit into an accepted classification scheme.

lesser degree, in the first two cytoplasmic loops. In general, the putative third cytoplasmic loops and the amino and carboxyl termini are the most divergent. Other members of this family of molecules that share sequence homology with the adrenergic receptors include receptors for 5-hydroxytryptamine (5-HT),<sup>59-61</sup> dopamine,<sup>62</sup> acetylcholine,<sup>63</sup> and light (opsins).<sup>28,64</sup> Other receptors, such as those for substance K,<sup>65</sup> the yeast  $\alpha$ - and  $\alpha$ -mating factors,<sup>66,67</sup> a chemoattractant receptor from the slime mold *Dictyostelium discoideum*,<sup>68</sup> as well as the *mas* oncogene product,<sup>69</sup> also share the same structural motifs but have little amino acid sequence homology with the adrenergic receptors. All of these macromolecules share other similarities that include one or more potential sites of extracellular N-linked glycosylation near the amino terminus, and several potential sites of phosphorylation in cytoplasmic domains that may be involved in the regulation of their function. The amino acid sequences of the adrenergic receptors cloned to date are presented in Figure 3.

The biological significance of the putative structural arrangement of these receptors is currently a matter of considerable conjecture. In fact, the pro-

posed arrangement for most of these receptors has been based solely on hydrophobicity plots and on analogy with bacteriorhodopsin.<sup>58</sup> The scarcity of most of the G protein-coupled receptors, which typically constitute 0.001% or less of total membrane protein, makes confirmation of these structural motifs difficult. The opsins are notable exceptions because they are the most abundant proteins in specialized bacterial membranes<sup>70</sup> and mammalian rod outer segments, which permits large scale protein purification schemes. Consequently, the presence of a bundle of seven transmembrane helices has been clearly established by high resolution electron diffraction for the bacteriorhodopsin of *Halobacterium halobium*.<sup>71</sup> A similar arrangement for vertebrate opsins has been supported by numerous techniques.<sup>63</sup> Many elements of this model have been confirmed for the turkey " $\beta_1$ -adrenergic receptor"<sup>72</sup> and hamster  $\beta_2$ -adrenergic receptor<sup>44</sup> using several approaches including limited proteolysis. However, it is clear that a number of strategies will be required to firmly establish the generality of the proposed model for other G protein-coupled receptors (e.g., antibodies recognizing specific receptor domains, electron diffraction, or x-ray

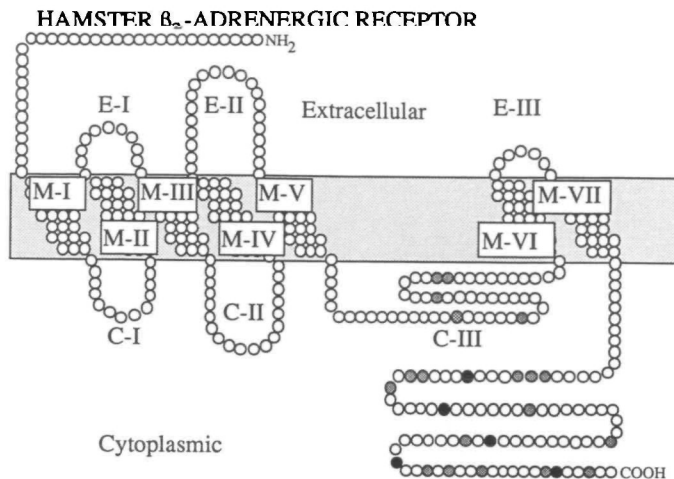


FIGURE 3. Schematic drawing of putative arrangement of the hamster  $\beta_2$ -adrenergic receptor within the cell membrane. Each circle represents an amino acid. The amino terminus and three loops (E-I, E-II, and E-III) are oriented extracellularly. Seven putative transmembrane  $\alpha$ -helices are represented as M-I through M-VII. Carboxyl terminus and three loops (C-I, C-II, and C-III) have a cytoplasmic orientation. Grey and black circles, respectively, represent serine and threonine residues that are potential sites of phosphorylation by  $\beta$ -adrenergic receptor kinase. This basic structural arrangement is likely to be shared by all of the cloned G protein-coupled receptors (see Figure 2).

crystallography). Analogy with the opsins has generated the hypothesis that the seven membrane-spanning helices may form a ligand-binding pocket, perhaps stabilized by disulfide bonds (discussed in the next section). Considerable evidence derived from studies of mutagenesis of the  $\beta_2$ -adrenergic receptor suggests that this arrangement may be critical for several receptor functions, including ligand binding and induction of conformational changes important for transducing intracellular signals via G proteins (see below).

On simple examination of the primary structure of the various G protein-coupled receptors characterized so far, certain structural motifs appear to be associated with receptors that are coupled to a particular signal transduction system. Receptors that are coupled to the inhibition of the enzyme adenylyl cyclase such as the  $\alpha_{2A}$ -adrenergic receptor ( $\alpha_{2C10}$ ) and  $\alpha_{2C4}$ -adrenergic receptor,<sup>16,36</sup> 5-HT<sub>1A</sub>,<sup>60</sup> dopamine D<sub>2</sub>,<sup>62</sup> and muscarinic M<sub>2</sub> and M<sub>3</sub> acetylcholine receptors<sup>63,73</sup> share short carboxyl termini and long third intracellular loops. Recent evidence obtained with eukaryotic expression systems suggests that  $\alpha_{2A}$ -adrenergic receptor ( $\alpha_{2C10}$ ),  $\alpha_{2C4}$ -adrenergic receptor,<sup>18</sup> 5-HT<sub>1A</sub>,<sup>27</sup> and muscarinic M<sub>2</sub> and M<sub>3</sub> receptors<sup>26,74,75</sup> are also capable of coupling weakly to phosphatidylinositol hydrolysis.

The  $\alpha_{1B}$ -adrenergic receptor,<sup>38</sup> 5-HT<sub>2</sub>,<sup>61</sup> 5-HT<sub>1C</sub>,<sup>59</sup> and substance K receptors<sup>65</sup> primarily stimulate phosphatidylinositol hydrolysis. These receptors have relatively long carboxyl termini and short third intracellular loops. The M<sub>1</sub> and M<sub>4</sub> muscarinic acetylcholine receptors seem to be an exception to this motif as they have relatively long third intracellular loops.<sup>59,73-76</sup> As expected, the carboxyl termini of the M<sub>1</sub> and M<sub>4</sub> receptors are longer than those of the  $\alpha_{2A}$ -adrenergic receptors,  $\alpha_{2A}$ -adrenergic receptors,<sup>16,36</sup> D<sub>2</sub>,<sup>62</sup> M<sub>2</sub> and M<sub>3</sub>,<sup>63,73</sup> and 5-HT<sub>1A</sub><sup>60</sup> receptors. However, they are shorter than those of the  $\alpha_{1B}$ -adrenergic receptors,<sup>38</sup> substance K, 5-HT<sub>2</sub>,<sup>61</sup> and 5-HT<sub>1C</sub><sup>59</sup> receptors.

A third structural motif is shared by receptors that primarily stimulate adenylyl cyclase or cGMP phosphodiesterase, such as the mammalian  $\beta_1$ -adrenergic receptor,  $\beta_2$ -adrenergic receptor, and  $\beta_3$ -adrenergic

receptor, the turkey  $\beta$ -adrenergic receptor, and the visual opsins.<sup>17,33-35,37,64</sup> All have long carboxyl termini that are relatively rich in serine and threonine residues and may serve as substrates for kinases such as the  $\beta$ -adrenergic receptor kinase, rhodopsin kinase, or related kinases. Taken together, these findings suggest that general secondary or tertiary conformational motifs of the G protein-coupled receptors may impart specificity to their linkage with second messenger systems. However, much more work will be necessary to delineate these relations in an unequivocal fashion. The known effector systems linked to the cloned adrenergic receptors are presented in Table 1. In the next several sections, the relation of specific regions of the adrenergic receptor molecules to their function will be discussed.

#### Functions of the Extracellular Domains

The extracellular domains of the G protein-coupled receptors consist of the three extracellular loops (E-I, E-II, and E-III) that connect the hydrophobic membrane-spanning stretches of amino acids and the glycosylated amino terminus<sup>44</sup> (see Figures 3 and 4). The role of the N-linked sugars of the amino termini of these proteins has not yet been established. However, several lines of evidence suggest that they are not involved in ligand binding.<sup>77-79</sup> Deglycosylated  $\beta_2$ -adrenergic receptor or  $\beta_2$ -adrenergic receptor translated without glycosylation by addition of tunicamycin to cultured cells, bind ligand normally.<sup>77,78</sup> Moreover, a mutant  $\beta_2$ -adrenergic receptor with a deletion of 10 amino acids from the amino terminus, including both potential sites of N-linked glycosylation, is nonglycosylated and binds ligand normally.<sup>79</sup> Thus, the functional role of the amino terminus and its sugar moieties is not currently known.

With respect to the extracellular loops, it has been suggested that cysteine residues within these domains may stabilize the ligand-binding pocket via disulfide linkages.<sup>40,41,79</sup> Comparison of the deduced amino acid sequences of various members of this receptor family (Figures 2 and 4) reveals a number of



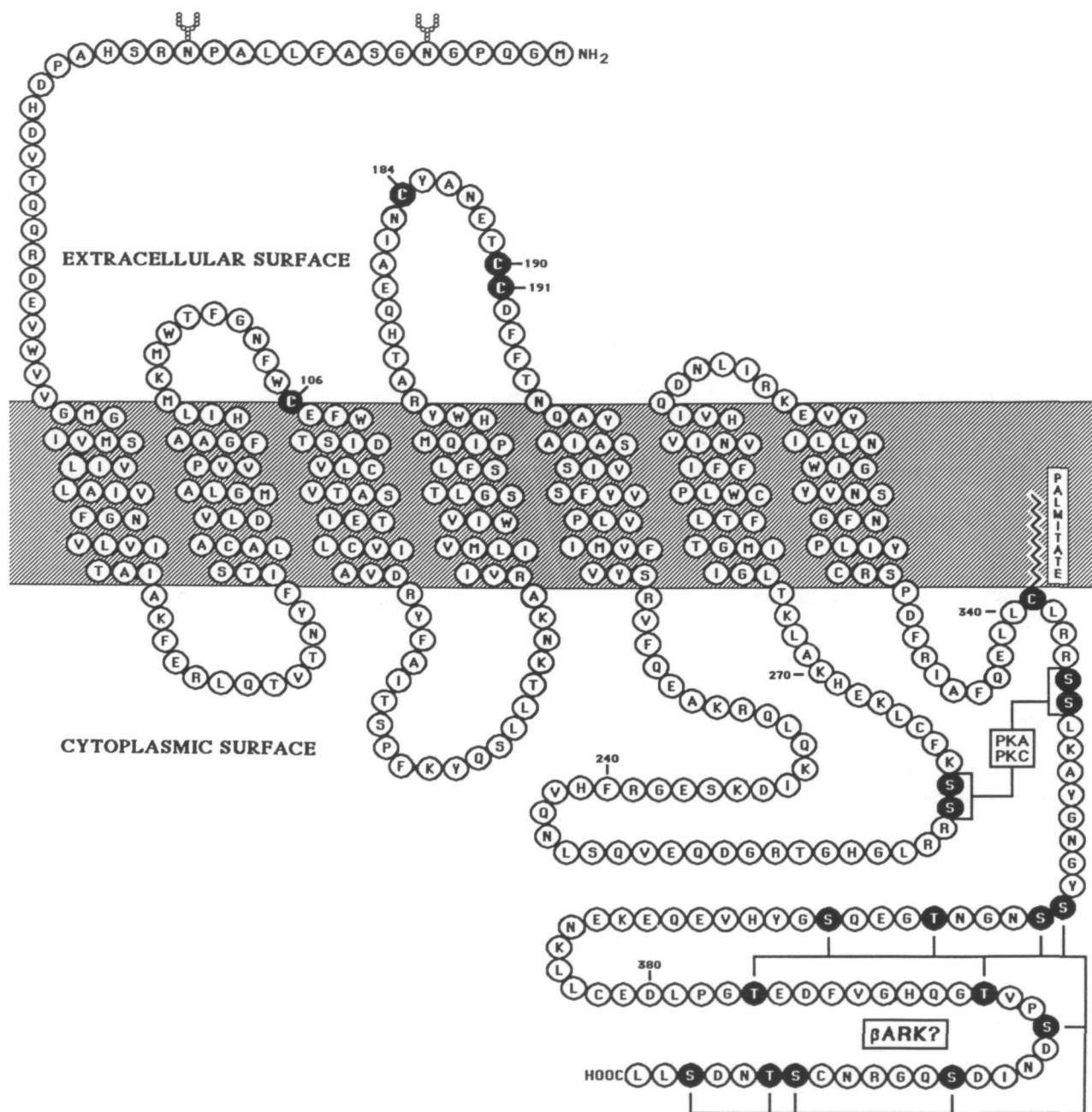


FIGURE 4. Posttranslational modifications of the human  $\beta_2$ -adrenergic receptor. Depicted are two sites of extracellular N-linked glycosylation (Asn<sup>5,16</sup>), four cysteine residues that may participate in disulfide bonds (Cys<sup>106,184,190,191</sup>), an intracellular cysteine residue (Cys<sup>341</sup>) that may serve as a site of attachment for a palmitate membrane anchor, as well as multiple threonine and serine residues located in C-III and the cytoplasmic carboxyl terminus, which are potential sites of phosphorylation by protein kinase A (PKA), protein kinase C (PKC), or  $\beta$ -adrenergic receptor kinase ( $\beta$ ARK).

highly conserved cysteine residues (Cys<sup>106,184</sup>, and Cys<sup>341</sup> of the human  $\beta_2$ -adrenergic receptor) that are potential candidates for disulfide bond formation. The role of cysteine residues of the  $\beta_2$ -adrenergic receptor has been systematically studied by site-directed mutagenesis. In an early study, ligand binding was drastically altered by substituting valine residues for Cys<sup>106</sup> and Cys<sup>184</sup>, two highly conserved extracellular cysteines.<sup>79</sup> These investigators hypothesized that Cys<sup>106</sup> and Cys<sup>184</sup> participate in a disulfide bond because alterations in

ligand-binding properties were not additive when both residues were substituted. More recently, Dohman et al<sup>41</sup> studied the effects of substitution of valine for the four extracellular cysteines (Cys<sup>106,184,190,191</sup>) and all four transmembrane cysteines (Cys<sup>77,116,125,285</sup>) of the human  $\beta_2$ -adrenergic receptor. Substitutions of any of the extracellular cysteines lowered ligand binding and altered binding specificity, whereas substitutions at any of the transmembrane cysteines had little effect. Fraser<sup>40</sup> has presented similar findings using

the mutagenesis approach. These results suggest that the four extracellular cysteines (Cys<sup>106,184,190,191</sup>) of the  $\beta_2$ -adrenergic receptor may participate in disulfide bonds that stabilize the binding pocket. Studies with rhodopsin suggest that this relation may apply to other members of the family of G protein-coupled receptors. Similar systematic examination of the role of the cysteine residues of bovine rhodopsin yielded similar results.<sup>80</sup> These findings are also consistent with previous chemical modification studies of rhodopsin.<sup>81,82</sup>

#### Transmembrane Domains are Important for Ligand Binding

The transmembrane domains of the adrenergic receptors consist of seven hydrophobic stretches of 20–28 amino acids. These are identified in Figure 2 as M-I through M-VII. The evidence suggesting that these conserved regions contain determinants critical for ligand binding has come from several experimental approaches. By covalently labeling the  $\beta_2$ -adrenergic receptor with a radioactive photoaffinity probe, followed by chemical and enzymatic digestion, Dohlmans et al<sup>44</sup> showed that a point of covalent attachment was to M-II of the hamster  $\beta_2$ -adrenergic receptor. This assignment was confirmed by direct amino acid sequencing, although the actual covalently labeled amino acid could not be identified. Another study showed that a similar photoaffinity probe incorporated into two tryptic fragments of the avian  $\beta$ -adrenergic receptor; one contained M-I through M-IV, and the other M-VII.<sup>72</sup> Using two different photoaffinity probes, Matsui et al<sup>83</sup> localized the covalent attachment site to the M-IV of the platelet  $\alpha_2$ -adrenergic receptor. Evidence derived from the study of muscarinic acetylcholine receptors also suggests that the transmembrane domains may be critical for ligand binding. It has recently been deduced that the alkylating agent [<sup>3</sup>H]PrBCM labels aspartate residues in M-II or M-III of the porcine brain muscarinic receptors.<sup>81</sup> These data fit the model proposed for rhodopsin in which the chromophore retinal is covalently attached to Lys<sup>296</sup>, which resides within M-VII.<sup>85,86</sup>

Using site-directed mutagenesis techniques to explore the role of the transmembrane regions of the  $\beta_2$ -adrenergic receptor in ligand binding, Dixon and coworkers<sup>79,87</sup> prepared a series of mutant hamster  $\beta_2$ -adrenergic receptors in which various regions of the molecule were deleted. When expressed in COS-7 cells, mutant  $\beta_2$ -adrenergic receptors with deletions of hydrophilic cytoplasmic or extracellular loops showed virtually no alterations in their ligand binding properties. However, deletions within the hydrophobic regions of the molecule ablated ligand binding.<sup>87</sup> Similarly, Kobilka et al<sup>55</sup> found that truncation of the carboxyl terminus of the human  $\beta_2$ -adrenergic receptor midway through M-VII destroyed ligand binding. Subsequently, it was shown that ligand-binding properties of the  $\beta_2$ -adrenergic receptor could be drastically altered by substituting single, highly conserved

amino acid residues in M-I, M-II, M-III, or M-VII.<sup>45,88,89</sup> These data strongly support the notion that determinants for agonist and antagonist binding lie within the transmembrane regions.

A third series of experiments using receptor chimeras has further underscored the importance of the transmembrane regions of the adrenergic receptor in determining ligand-binding properties. By using a series of human  $\beta_1$ - $\beta_2$ -adrenergic receptor<sup>48</sup> and  $\alpha_2$ - $\beta_2$ -adrenergic receptor<sup>49</sup> chimeras, M-VI and M-VII were shown to be most important in conferring antagonist binding specificity. Although the determinants for agonist binding overlapped with those for antagonist binding, they were less distinct and clearly not identical. These results are not surprising in view of the proposed G protein-coupled receptor structural model. If M-I through M-VII form a ligand-binding pocket, the amphipathic  $\alpha$ -helical arrangement could allow hydrophobic residues to be oriented toward the membrane lipids, while certain critically charged amino acids can be oriented toward the aqueous inner surface to form a binding pocket. Such a geometry would create many distinct potential sites for interaction with agonists and antagonists. Thus, each ligand might be positioned in the binding pocket in a slightly different manner. Pharmacological studies of various receptors further support this notion. For example, the human  $\beta_2$ -adrenergic receptor has a low affinity for 5-HT, and the human 5-HT<sub>1A</sub> receptor has a low affinity for catecholamines. However, both receptors share high-to-moderate affinities for certain antagonists such as cyanopindolol and propranolol.<sup>60,90</sup> These two receptors share  $\approx 75\%$  sequence identity within M-VI and M-VII. Another comparison can be made between the human 5-HT<sub>1A</sub> receptor<sup>60</sup> and a rat brain dopamine D<sub>2</sub> receptor.<sup>62</sup> Each receptor exhibits a low affinity for the other's endogenous agonist, but share high affinities ( $\approx 1$ – $50$  nM) for compounds such as lysergic acid diethylamide, lisuride, (+) butaclamol, spiroxatrine, metergoline, and buspirone.<sup>91</sup> These receptors share  $\approx 50\%$  sequence identity within M-II, M-III, M-VI, and M-VII. Clearly, there is a complex relation between the amino acid composition of the transmembrane regions and ligand-binding properties of the G protein-coupled receptors.

#### Cytoplasmic Domains are Important for Receptor-G Protein Interactions

The cytoplasmic domains of the G protein-coupled receptors comprise the three intracellular loops (C-I, C-II, and C-III) and the carboxyl terminus (see Figures 2–4). These regions may be critical for many receptor functions such as G protein coupling, phosphorylation reactions, and desensitization. We will discuss the functions of each of the cytoplasmic domains separately, but their functions may overlap considerably. In this regard, two regions, C-III and the amino terminal portion of the carboxyl terminus, are the best characterized. It has been firmly established that C-III contains sequences important for

receptor-G protein coupling. Initially, mutant hamster  $\beta_2$ -adrenergic receptors, which lacked C-III, were shown to have greatly diminished ability to stimulate adenylyl cyclase, suggesting that this region of the receptor was important in G protein coupling.<sup>42,87</sup> The carboxyl end of C-III has been shown to be particularly important. The N-terminal region of C-III may also participate in G protein coupling but in a more general fashion<sup>42,47</sup>; however, evidence to date indicates that this region may not contain the major determinant of specificity for the interaction of receptor with G proteins.<sup>49</sup> Using  $\alpha_2$ - $\beta_2$ -adrenergic receptor chimeras, Kobilka et al<sup>49</sup> demonstrated that  $\alpha_2$ -adrenergic receptor, into which 80 or 122 amino acids of the  $\beta_2$ -adrenergic receptor sequence had been inserted, could mediate stimulation of adenylyl cyclase, albeit to a lesser degree than wild-type  $\beta_2$ -adrenergic receptor. These  $\alpha_2$ - $\beta_2$ -adrenergic receptor chimeras contained the entire C-III segment, 21 amino acids from the M-VI segment, and either the entire M-V and E-II or 7 amino acids from M-V of the human  $\beta_2$ -adrenergic receptor. The former chimera mediated stimulation of adenylyl cyclase more efficiently than the latter suggesting that portions of M-V influence G protein coupling. This influence is either due to a direct participation in receptor-G protein coupling or to secondary effects on conformation. Similar results have been obtained with a mutant rhodopsin, where substitution of Lys<sup>248</sup> at the carboxyl end of rhodopsin C-III greatly decreased the ability of the molecule to couple to transducin, the G protein primarily coupled to opsins.<sup>92</sup> In a similar fashion, the analogous vicinal basic amino acid residues at the carboxyl end of C-III (i.e., His<sup>269</sup> and Lys<sup>270</sup>) of the human  $\beta_2$ -adrenergic receptor may play a critical role in receptor coupling to G proteins (M. Hnatowich, B.F. O'Dowd, M.G. Caron, R.J. Lefkowitz, unpublished observations).

Another cytoplasmic region thought to be important for receptor-G protein coupling is the N-terminal region of the carboxyl termini of both the  $\beta_2$ -adrenergic receptor<sup>42</sup> and rhodopsin.<sup>93</sup> A cysteine residue (Cys<sup>341</sup> of the  $\beta_2$ -adrenergic receptor) conserved in most G protein-coupled receptors characterized so far may be of particular importance (see Figure 4). O'Dowd et al<sup>39</sup> have recently demonstrated that Cys<sup>341</sup> of the human  $\beta_2$ -adrenergic receptor is palmitoylated, and that substitution of this residue results in a nonpalmitoylated form of the receptor. This mutant demonstrates a marked reduction in receptor-mediated stimulation of adenylyl cyclase. In the case of rhodopsin the analogous cysteine residues in this region (Cys<sup>322</sup>, Cys<sup>323</sup>) have been suggested to form a disulfide bridge<sup>92</sup> or to undergo fatty acylation with palmitic acid.<sup>93</sup> It has been speculated that palmitoylation of both rhodopsin and  $\beta_2$ -adrenergic receptor may anchor this region of the molecule to the plasma membrane in such a way as to present a favorable conformation for G protein coupling, thus essentially forming a fourth intracellular loop (Figure 4).

The functions of other cytoplasmic domains are less well characterized. Mutations of C-I of the  $\beta_2$ -adrenergic receptor result in very poor receptor expression in transient expression systems, possibly due to defective protein folding during assembly, defective membrane insertion, or both.<sup>42,47,55,79</sup> Mutations of the carboxyl end of C-II appear not to affect receptor function, whereas the integrity of the amino portion of C-II, particularly the highly conserved proline (Pro<sup>138</sup> of human  $\beta_2$ -adrenergic receptor), may be essential for receptor-G protein coupling.<sup>42,47</sup> Pro<sup>138</sup> may either interact directly with the G protein or contribute to coupling efficiency through an indirect mechanism, such as conferring the proper alignment of C-II with respect to other regions of the receptor, which more directly interact with the G protein. Direct evidence, however, will have to await further studies.

The cytoplasmic domains of the  $\beta_2$ -adrenergic receptor may also be involved in various events that lead to receptor desensitization.<sup>46,47</sup> This aspect of receptor function will be discussed in the next section.

#### Functional Regulation of Adrenergic Receptors

The functions of G protein-coupled receptors are subject to dynamic regulation by a number of mechanisms. Covalent modification of the receptor (i.e., phosphorylation by various kinases) has been implicated in the regulation of  $\beta$ -adrenergic receptor function.<sup>19</sup> Phosphorylation of  $\beta$ -adrenergic receptor is closely associated with impaired receptor function,<sup>96-98</sup> correlating with a decreased ability to couple to its G protein  $G_i$ . The paradigm in which receptor phosphorylation reactions have been examined is the phenomenon known as desensitization. Desensitization refers to the attenuation of responsiveness to a drug or hormone in its continued presence. This phenomenon can markedly diminish the therapeutic efficacy and duration of action of a drug. In the realm of hypertension, the rapid tachyphylaxis associated with the use of the potent antihypertensive trimethaphan camsylate may be a clinically important example of this phenomenon.

Several distinct patterns of phosphorylation of adrenergic receptors have been correlated with different aspects of receptor desensitization. These patterns differ with respect to their temporal occurrence as well as to their dependence on various kinases or agonist occupancy. Two types of phosphorylation reactions dependent on the presence of agonists can lead to the two classical types of desensitization (heterologous and homologous). Homologous desensitization refers to an attenuation of responsiveness to a single type of agonist after exposure of the receptor to that particular agent. Heterologous desensitization differs in that exposure of a cell to a particular agonist brings about attenuation of responsiveness to multiple agonists operating through distinct receptors. In many systems, heterologous desensitization can occur concomitantly with homologous desensitization.



The best characterized example of heterologous desensitization *in vivo* is a feedback regulation of  $\beta$ -adrenergic receptors associated with phosphorylation by PKA.<sup>99,100</sup> Thus, when adenylyl cyclase is stimulated by any number of hormones, intracellular levels of cAMP rise, PKA is activated, and receptors are phosphorylated and desensitized. *In vitro*, the rate, but not the extent, of phosphorylation of  $\beta$ -adrenergic receptor by PKA is increased by receptor occupancy.<sup>98</sup> Functional impairment of components of the signal transduction network other than the receptor may also participate in heterologous desensitization. Several groups have explored the role of G protein modification in heterologous desensitization. Kassis and Fishman<sup>101</sup> demonstrated that  $G_i$  derived from fibroblasts that exhibited heterologous desensitization after exposure to prostaglandin  $E_1$  was functionally impaired when reconstituted into *cyc*<sup>-</sup> S49 lymphoma cell membranes, but this situation may not pertain to every system examined (reviewed in Reference 102). Evidence has also suggested that the inhibitory G protein  $G_i$  may participate in heterologous desensitization.<sup>103,104</sup> Recent data suggest that PKA or PKC can phosphorylate the catalytic subunit of adenylyl cyclase in broken cell membrane preparations<sup>105</sup> and in whole cells in culture<sup>106</sup> leading to an impairment<sup>105</sup> or enhancement<sup>105,107</sup> of adenylyl cyclase activity. However, the physiological relevance of these phosphorylation reactions is not presently known.

Homologous desensitization should by definition be simpler mechanistically as it is hormone specific. Nonetheless, several distinct processes also appear to contribute to this form of desensitization: 1) receptors are rapidly uncoupled from the effector systems, 2) receptors are rapidly sequestered or internalized, and 3) receptors are slowly down-regulated. The exact mechanisms by which these processes mediate homologous desensitization are not totally understood. Current evidence indicates that the rapid onset of desensitization involves the phosphorylation of the  $\beta_2$ -adrenergic receptor by a specific kinase. A protein kinase,  $\beta$ -adrenergic receptor kinase ( $\beta$ ARK) capable of phosphorylation of the  $\beta_2$ -adrenergic receptor in the presence of agonist has been identified and characterized.<sup>97,108-111</sup> This kinase phosphorylates the agonist-occupied form of the  $\beta_2$ -adrenergic receptor on serine and threonine residues in the carboxyl terminus of the receptor.<sup>46,108</sup> Phosphorylation of the receptor by  $\beta$ ARK clearly decreases  $\beta_2$ -adrenergic receptor function, although one or more proteins or other cofactors may be necessary to fully express this effect.<sup>111</sup> The effects of  $\beta$ ARK on the  $\beta_2$ -adrenergic receptor are remarkably analogous to those of the retinal enzyme rhodopsin kinase, which phosphorylates rhodopsin in a light-dependent fashion.<sup>112</sup> Rhodopsin kinase phosphorylates multiple threonine and serine residues in the carboxyl terminus and at least one threonine in the third intracellular loop of rhodopsin. This process is associated with a decreased ability of rhodopsin to

couple to transducin, its G protein. This process is dependent on subsequent binding to arrestin (48 kDa protein) to the phosphorylated rhodopsin.<sup>112</sup> The prospect that an analogous 48 kDa-like protein may exist that participates with  $\beta$ ARK in the regulation of the  $\beta_2$ -adrenergic receptor was suggested in a recent study.<sup>111</sup>

Regarding the specificity of  $\beta$ ARK, Benovic et al<sup>113</sup> have demonstrated that the human platelet  $\alpha_2A$ -adrenergic receptor is an excellent substrate for  $\beta$ ARK in an *in vitro* reconstitution system. The phosphorylation of the  $\alpha_2A$ -adrenergic receptor is entirely agonist dependent. Although the physiological relevance of this interaction is currently not known, the potential interaction of  $\beta$ ARK with receptors other than  $\beta_2$ -adrenergic receptor raises an intriguing question. How can  $\beta$ ARK mediate "agonist-specific" homologous desensitization when it potentially phosphorylates many distinct receptors? A clue lies in the agonist-dependent nature of  $\beta$ ARK-mediated receptor phosphorylation. In this sense, agonist occupancy of receptors can confer a specificity of sorts. Agonist binding presumably induces a conformational change in the receptor, exposing previously unavailable  $\beta$ ARK substrate sites on the cytoplasmic domains of the receptor. The recent cloning of the gene for  $\beta$ ARK<sup>114</sup> should allow this question to be specifically addressed.

Recent experiments using site-directed mutagenesis of the  $\beta_2$ -adrenergic receptor gene have delineated the regions of the receptor involved in the regulatory phosphorylation. These experiments have also established a role for receptor phosphorylation in the process of rapid desensitization. There are two consensus sequences for phosphorylation of the receptor by PKA, one in the third cytoplasmic loop and another in the amino terminal part of the carboxyl tail. In contrast, the putative sites of  $\beta$ ARK-induced phosphorylation appear to reside primarily in a serine- and threonine-rich region toward the distal part of the carboxyl terminal of the receptor. When either of the PKA consensus sequences or the presumed  $\beta$ ARK phosphorylation sites of the receptor are altered by site-directed mutagenesis, a marked decrease in the ability of agonists to mediate receptor phosphorylation is observed. This is corroborated by a marked slowing and attenuation of the desensitization process.<sup>46,56</sup> These effects can be demonstrated by examining whole cell cAMP or hormone-stimulated adenylyl cyclase in membranes.<sup>56,57</sup> These observations delineate the sites of covalent modification of the receptor by these kinases and implicate these mechanisms in the process of desensitization.

To date, virtually nothing is known of the structural regions of the receptors that are involved in mediating receptor sequestration. Although it has been contended by Cheung et al<sup>115</sup> that those regions of the third cytoplasmic loop of the  $\beta_2$ -adrenergic receptor that are involved in mediating coupling to G proteins (see above) are also involved in mediating

receptor sequestration, recent studies in our laboratory do not support this contention. Thus, a variety of receptor mutants that are not capable of coupling to  $G_i$  are nonetheless able to be sequestered normally (P. Campbell, M. Hnatowich, W.P. Hausdorff, M. Bouvier, B.F. O'Dowd, M.G. Caron, R.J. Lefkowitz, unpublished observations).

The structural basis for receptor down-regulation is poorly understood but is a subject of current investigation. There appears to be both cAMP-dependent and cAMP-independent mechanisms that lead to down-regulation. The mechanisms are likely to be complex and involve alterations in the rates of receptor degradation or assembly or processing, messenger RNA (mRNA) stability, and alterations in the rates of receptor gene transcription. In this connection, receptor mutants that cannot be phosphorylated by PKA showed a slowed rate of cAMP-induced receptor down-regulation.<sup>116,117</sup> The attenuated rate of receptor down-regulation is likely due to an alteration in the rates of receptor degradation. Additionally, it has recently been shown that cAMP stimulates transcription of the  $\beta_2$ -adrenergic receptor gene and increases the steady-state levels of  $\beta_2$ -adrenergic receptor mRNA in DDT-1 MF<sub>2</sub> cells.<sup>118</sup> Clearly, much more work will be necessary to unravel the very complex mechanisms that control receptor down-regulation.

G protein-coupled receptors are involved in the responsiveness of numerous tissues and cells to a variety of stimuli. Adrenergic receptors have served as models for the study of the relations between structure and function of G protein-coupled receptors. The cloning of the genes or cDNAs for the various subtypes of adrenergic receptors has greatly enhanced our understanding of the cellular and biochemical actions of the catecholamine hormones. In the broadest possible sense, these receptors serve as excellent models for the study of the general mechanisms of all G protein-coupled receptors. Model systems using the cloned adrenergic receptors will also undoubtedly prove critical to the resolution of more specific problems, such as the controversy regarding the relative importance of  $\alpha$ -receptor subtypes in genetic hypertension in rats.<sup>119,120</sup> Elucidation of the functional principles of these receptors should lead to a better understanding of the mechanisms involved in regulating hormone and drug responsiveness as well as the molecular defects underlying certain diseases.

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